MICRO-MORPHOLOGICAL STUDIES ON THE DEGRADATION OF LIGNIFIED CELL WALLS BY ASCOMYCETES AND FUNGI IMPERFECTI

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Introduction

In recent years it has been found that lower Ascomycetes and Fungi Imperfecti may be responsible for severe decay of timber under certain conditions, particularly in very wet situations. This type of fungal decay, which Savory (1954) has termed 'soft rot', is generally recognised by the macroscopic appearance of the wood, the superficial layers being extremely soft when wet and cracking across the grain when dry. Microscopically, wood severely attacked by soft rot fungi has a distinctive appearance. Transverse sections of decayed wood show large numbers of small circular areas of erosion within the central layer of the secondary wall, while in longitudinal section the eroded areas are seen to consist of a series of cavities with conical ends (Savory 1954, Duncan 1960). Bailey and Vestal (1937) observed similar phenomena during the course of anatomical studies on wood and showed that the cavities were aligned in the same spiral arrangement as the cellulose microfibrils.

Courtois (1963) made detailed observations on the shape and size of the cavities in soft-rotted wood. There is little information on the mechanism of fungal penetration of the secondary wall or on the relation between hyphae and soft rot cavities. Duncan (1960) has published a photo-micrograph which shows a hypha lying in a cavity in the wall of a fibre-tracheid of birch wood taken from a water-cooling tower and Meier (1955) and Liese (1963) have attempted to study the relationship using the electron microscope. The main purposes of the present investigation were to observe the early stages of hyphal penetration of the secondary wall by a single fungal species known to cause the particular type of wall degradation described above, and to compare the mode of attack of different species of wood by a number of active fungal species. It was planned to carry out both sets of experiments in pure culture under controlled conditions in the laboratory.

Materials and Methods

Two series of experiments were performed. (1) To determine the effect of the Ascomycete *Chaetomium globosum* Kunz* on wood of one coniferous species, Scots pine (*Pinus sylvestris* L. and one broad-leaved species, birch (*Betula* sp.). (2) To determine the effect of six species o Fungi Imperfecti on birch. These species were *Camarosporium ambiens**, *Cephalosporiur* sp.,* *Coniothyrium fuckelii* Sace.,* *Phoma* sp.,* *Sphaeronema* sp.,* *Stysanus stemoniti* (Pers.) Cda.* All species had been isolated from Scots pine or birch fence posts showin typical symptoms of soft rot (i.e. cavities within the wall) (Corbett and Levy, 1963).

Experimental Method

Small blocks of sapwood (0·7cm^3) were cut carefully so that the three opposite pairs of fac were in the transverse, tangential longitudinal and radial longitudinal planes. The faces of t

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**The fungal species will subsequently be referred to by their generic name only.*

block were sealed off with a layer of 'Araldite' (a proprietry formulation of an epoxy-resin adhesive) leaving one pair of opposite faces unsealed. The blocks were sterilised by placing them in a muslin bag, which was suspended in steam for 30 minutes and then placed in an atmosphere of propylene oxide for 24 hours. At the end of this period the bag was aerated under sterile conditions to remove residual propylene oxide. Pure cultures of the test fungi were prepared on agar plates in petri dishes. The agar contained mineral nutrients (after Abrams, 1948) and, glucose (0·1 per cent). In some experiments Scots pine blocks were placed on cultures growing on malt agar as described in an earlier report (Corbett and Levy, 1963a). Under these conditions decay was less rapid than on Abrams agar. The blocks were placed on vegetative mycelium with one unsealed face in contact with the mycelium and incubated at 25°C. The period of incubation was varied from 1—6 weeks according to the susceptibility of the wood, the exposure period being longer for the more resistant coniferous wood. At harvest the blocks were fixed in formalin-acetic acid-alcohol (formalin 6 c.c., glacial acetic acid 4 c.c., 70 per cent ethanol 90 c.c.). Sections ranging from 5μ to 25μ were cut directly from the blocks on a sledge microtome and stained with safranin and picro aniline blue (Cartwright 1929). Radial longitudinal sections were found to be most suitable for the examination of hyphal activity on the cell walls. The xylem and ray tracheids of Scots pine and xylem fibre-tracheids of birch were examined for evidence of decay.

In the second series of experiments the procedure differed from this in that the faces were unsealed and the tangential face placed in contact with the fungal culture.

Results

(1) EXPERIMENTS WITH *CHAETOMIUM GLOBOSUM*

(a) *The mode of cell wall destruction*

Two morphologically distinct types of decay were observed in these experiments. The first type was characterised by the penetration and subsequent establishment of hyphae within the secondary wall giving rise to cavities of the type described by previous workers (e.g. Bailey and Vestal 1937, Savory 1954). In the second type of decay, there was a general dissolution of the wall from the lumen by hyphae lying in contact with the inner surface (birch only).

Type 1. The development of this decay in tracheid walls of Scots pine infected by *Chaetomium* was studied in detail. Hyphae invade the tracheid lumen and align themselves parallel to the long axis, usually contiguous with the wall. Hyphal diameter at this stage is 1-3μ. These hypha develop lateral branches which penetrate the wall (Fig. 1). Within the wall each penetrating hypha becomes markedly reduced in diameter (*ca.* 0·5μ) and forms a bore-hole very little wider than itself at right angles to the long axis of the tracheid. (Fig. 2.) This cavity never increases in diameter. Such lateral hyphae frequently pass completely through the walls of the two adjoining tracheids in a manner similar to the action of sap-stain fungi such as *Diplodia* spp. (Cartwright and Findlay, 1958 Plate 56). The hyphae resume their original form on reaching the lumen of the second wall. This stage was found to be indicative of incipient decay. Some lateral hyphae branch vertically on reaching the S_2 layer of the second tracheid (Fig. 3). The terminology used to designate the layers (S_1, S_2, S_3) of the secondary wall follows that used by Roelofsen (1959), after Kerr and Bailey (1934). The upper and lower arms of the T-shape thus formed grow at approximately the same rate and at a slight but definite angle to the long axis of the tracheid. Close examination of these branches suggests that the type of

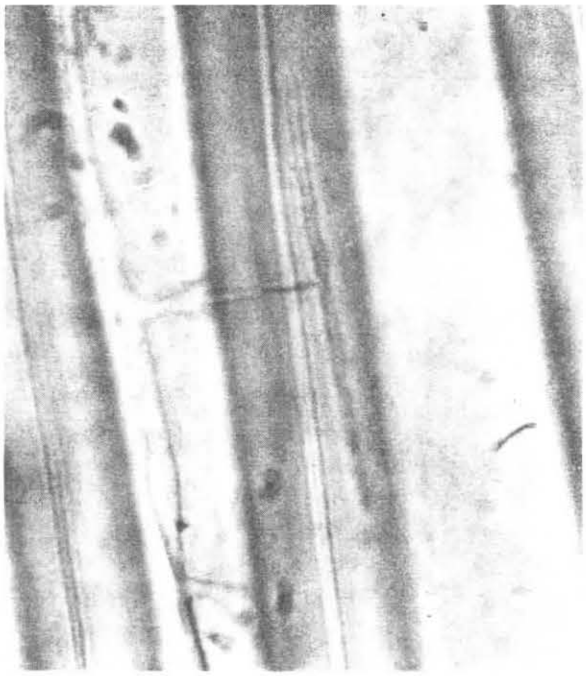

Figure 1 Hypha of *Chaetomium globosum* forming lateral branch which penetrates wall of tracheid of *Pinus sylvestris*. x 1500.

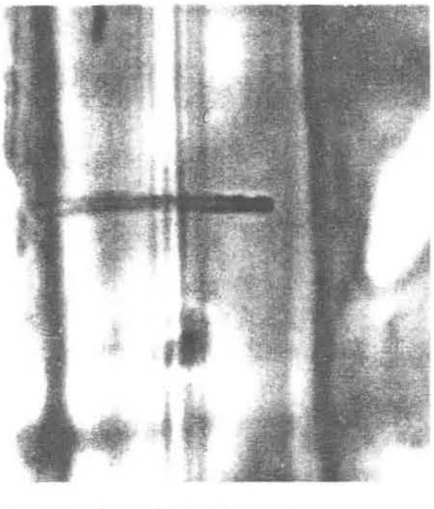

Figure 2 Initial penetration of lateral branch of hypha of *C. globosum* into tracheid wall of *P. sylvestris* showing hypha completely filling the cavity. x 1500.

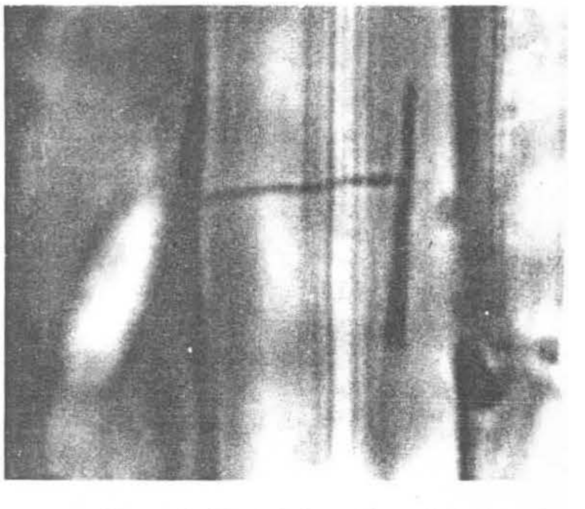

Figure 3. Vertical branching of hypha of *globosum* in S_2 layer of wall of adjacent trache of *P. sylvestris*. x 1500.

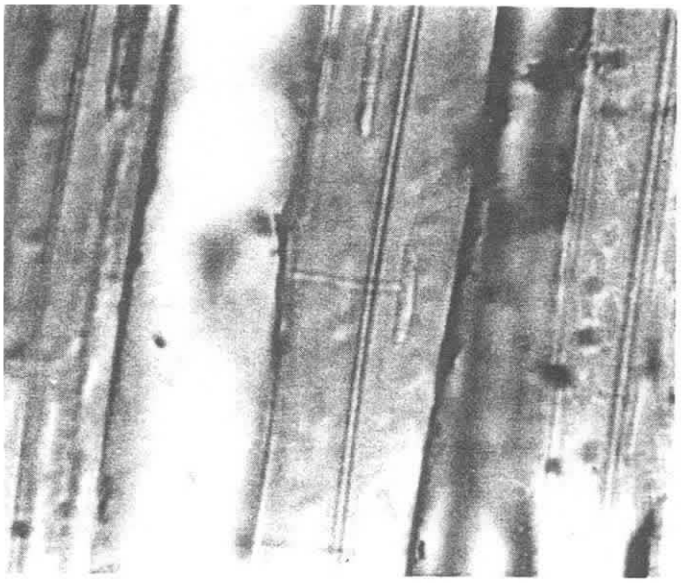

Figure 4 Sharply angled tips of cavity formed by hypha of *C. globosum* in S_2 layer of tracheid wall of *P. sylvestris*. x 1000.

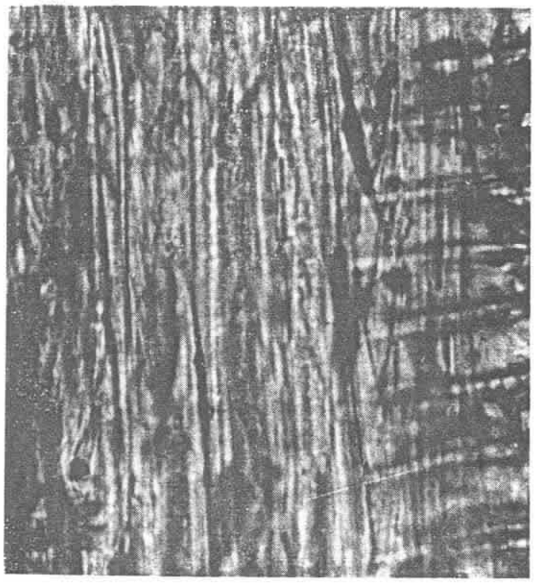

Figure 5 'Proboscis' hyphae initiating further cavities. x 1000.

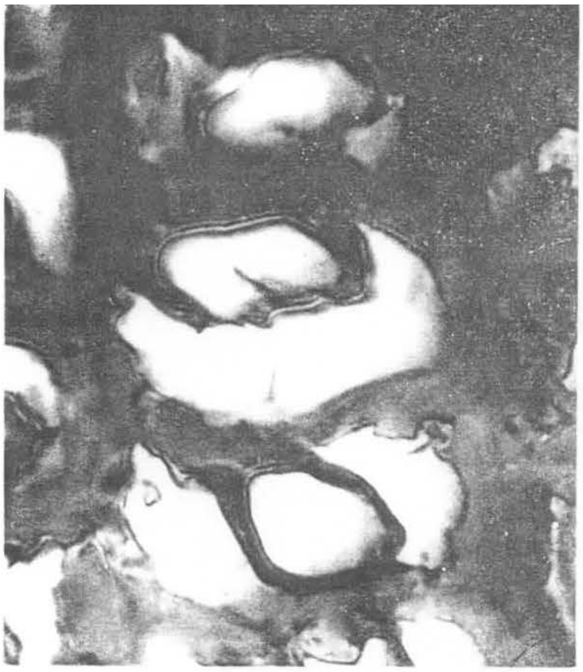

Figure 6 Resistant S_3 layer of *P. sylvestris* tracheid wall remaining after destruction of S_2 layer by *C. globosum*. x 1500.

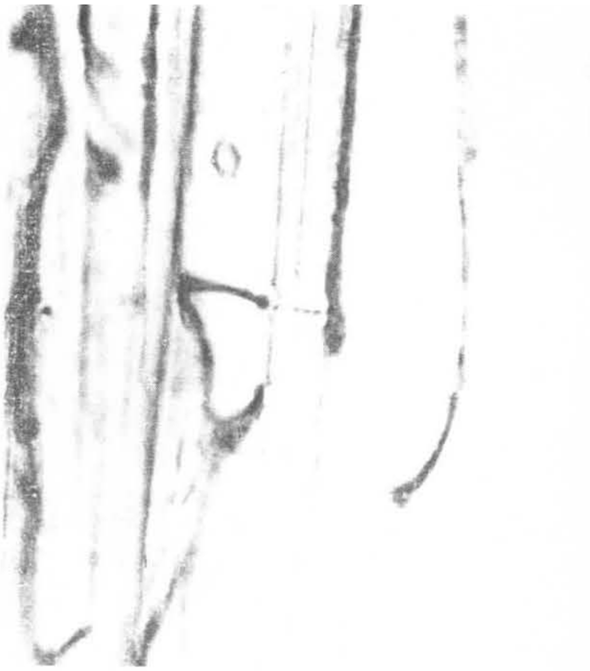

Figure 7 V-shaped erosion of wall by hypha tip prior to penetration of the wall. *C. globosum* in *Betula sp.* x 1000.

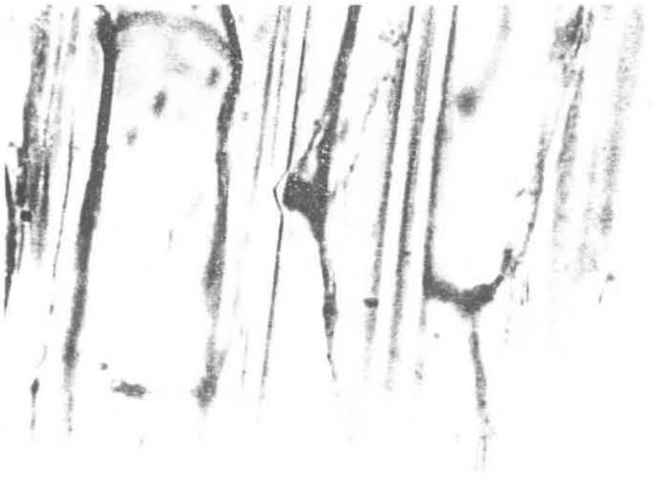

Figure 8 Sinuous erosion of the lumen by a hypha of *C. globosum* in *Betula sp.* x 1000.

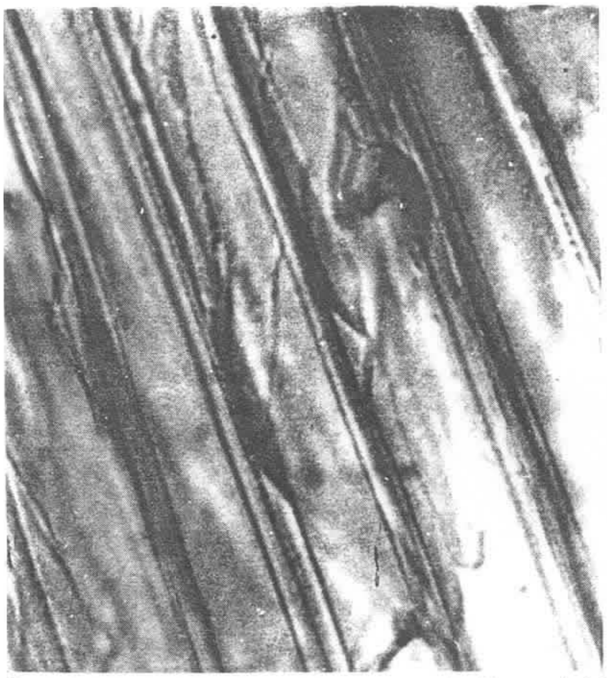

Figure 9 Erosion troughs formed in the wall of a fibre tracheid of *Betula sp.* by hypha of *C. globosum.* x 1000.

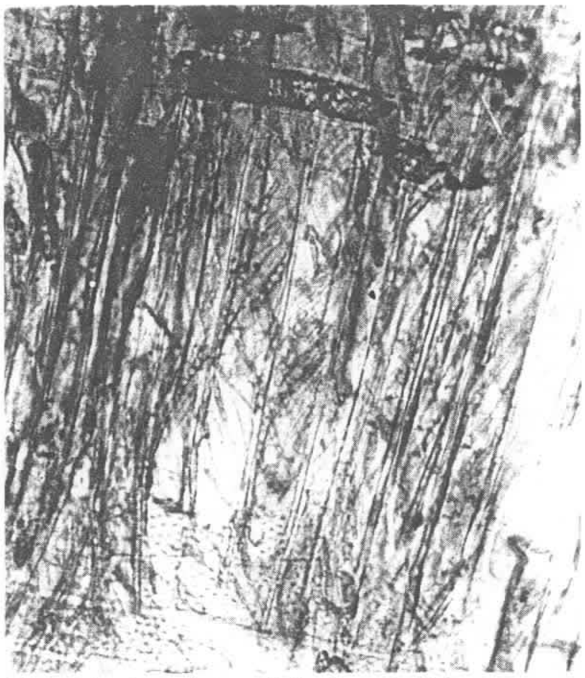

Figure 10 Degradation of *Betula sp.* by *Sphaeronema sp.* x 240.

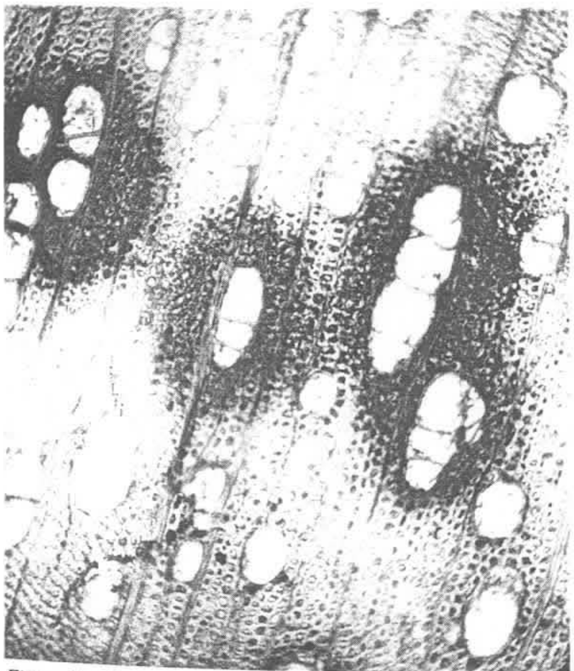

Figure 11 Degradation of *Betula sp.* by *Stysanus stemonitis.* x 60.

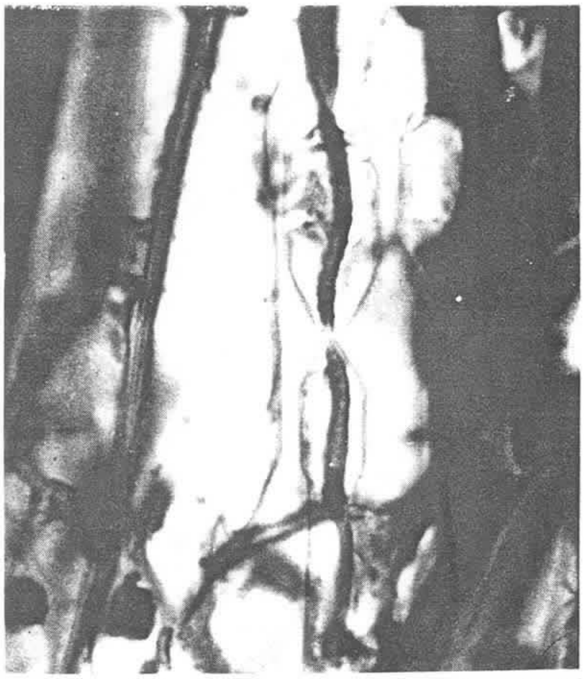

Figure 12 Hypha of a soft rot fungus lying in the centre of cavities in the wall of a fibre-tracheid of *Betula sp.* x 1500.

branching is falsely dichotomous with the lateral hypha changing its direction by 90° and simultaneously giving off a branch in the direction opposite to that of the new growth. Subsequently, the rate of vertical penetration is reduced and the hyphae enlarges the cavity in a lateral direction within the S_2 layer. At this stage the tips of the cavity taper (Fig. 4) giving rise to sharply angled constrictions. Further cavities are initiated by a fine hypha growing from the apex of the constriction or by fine lateral hyphal branches from a hypha in an existing cavity. (Fig. 5.) The distance from the point of vertical branching to the apex of the first constriction and from one point of maximum constriction to the next is variable but appears to be related to the diameter of the cavity – the smaller the cavity diameter the greater the distance between constrictions. General dissolution of the S_2 layer occurs in advanced stages of rot but the S_3 layer is not attacked (Fig. 6). In laboratory experiments the cavities eroded by *Chaetomium* in Scots pine summer wood tracheids were of large diameter (mean diameter 12 μ) and almost axially orientated. In the ray tracheids cavities were occasionally noted and were generally smaller in diameter (mean diameter 3·5 μ).

Type 2. This type of decay was not observed in Scots pine. Morphologically, it was a much simpler type of decay than Type I and was brought about by a hypha in the lumen coming in contact with the wall of a fibre tracheid of birch. In some cases a lateral branch with a slightly bulbous tip produced a fine hyphal thread which eroded the wall initially giving a V-shaped figure (Fig. 7). More commonly erosion of the wall occurred along the length of any hypha in contact with the wall. When seen in longitudinal face view this erosion appeared as a sinuous figure following the path of the hypha (Fig. 8) but in longitudinal section it was seen to be a sharp-sided trough-shaped figure (Fig. 9). Both V and trough-shaped eroded areas penetrated the secondary wall as far as the S_1 layer or the primary wall-middle lamella complex. Fine hyphae passed across the wall at right angles to the long axis connecting V-shaped eroded regions in adjoining fibre-tracheids. The sharp angles of the erosion figures as seen in longitudinal section were less obvious in transverse section.

(b) *The effect of the nature of the exposed face on decay*

The structure of the exposed face affected both the initial susceptibility to attack (both wood species) and the type of attack (birch).

Scots pine

In Scots pine it was found that susceptibility was independent of the rapidity with which the fungus spread through the block. Hyphae of *Chaetomium* had spread throughout blocks in all three treatments and formed perithecia on the upper unsealed face after 12 days exposure. The greatest production of perithecia occurred on transverse (blocks having a transverse, radial or tangential face exposed are referred to henceforth as 'transverse', 'radial' or 'tangential' blocks respectively) blocks, the least on radial blocks. No decay had occurred at this stage. After six weeks perithecia production on the upper surfaces followed the same pattern as after 12 days and decay symptoms were observed in radial blocks but not in transverse or tangential blocks. In radial blocks the cells most susceptible to attack by *Chaetomium* were the tracheids on the exposed face although tracheids associated with rays within the block showed many immature cavities after five weeks. In these blocks hyphae proliferated in the ray parenchyma, passed into the ray tracheids through the semi-bordered pits, and then attacked the walls of adjacent xylem tracheids. Lateral spread of hyphae across the radial face through the ray parenchyma was rapid whereas tangential spread was restricted with the hyphae passing from tracheid to tracheid through the pits. In all three block types the ray tissue showed the densest growth of hyphae at the end of the exposure period, and this tissue appeared to establish a reservoir of hyphae from which further attack could proceed at some distance from the exposed surface. Examination of sections taken from Scots pine fence posts confirmed the observation that rot proceeds from the rays at a distance from the surface. In tangential blocks hyphae spread rapidly through the ray parenchyma but no decay was observed after six weeks.

Birch

Chaetomium hyphae had spread completely through all birch blocks after eight days and were distributed equally in vessels, fibre-tracheids and ray parenchyma. At this stage perithecia production was greatest on transverse blocks and least on radial blocks as in Scots pine. However, in contrast to Scots pine, transverse blocks decayed rapidly, all edges of the block showing well-developed lumen erosion characteristic of the second type of decay (Type 2) after six days. In tangential blocks decay was again of Type 2 but was confined to the exposed edge, where it occurred to a depth of approximately 0·5mm. (= diameter of 80 fibre-tracheids). The depth of decay was even less in radial blocks, only the peripheral 3—5 fibre-tracheids of the exposed face being attacked. Moreover in these blocks the decay took the form of discrete spiral cavities as produced in the first decay category (Type 1). After 24 days transverse blocks were very soft and examination of sections showed that only a skeletal framework made up of the primary wall—middle lamella complex of the fibre-tracheid walls remained. Tangential blocks showed severe lumen erosion to a depth of 1·5 mm. and radial blocks showed spiralling cavities to a depth varying from 6—40 fibre-tracheids from the exposed face. For birch therefore the structure of the exposed face affected not only the degree of attack by *Chaetomium* but also the type of attack, and it is evident that for birch the presence of hyphae in the lumen of fibre-tracheids is an important prerequisite for attack.

(2) EXPERIMENTS WITH FUNGI IMPERFECTI

Pronounced decay developed in birch blocks after three weeks exposure to *Coniothyrium*, *Sphaeronema* or *Stysanus*. *Coniothyrium* attacked tangential blocks in the same manner as *Chaetomium* although the activity of individual hyphae of *Coniothyrium* in destroying the cell wall was observed to be greater than for *Chaetomium*. The attack of tangential blocks was similar to that of *Chaetomium*, destruction of the wall being chiefly by lumen erosion. On the other hand *Sphaeronema* (Fig. 10) and *Stysanus* (Fig. 11) formed steep-angled spiralling cavities within the walls of fibre-tracheids in patches throughout the block. Early stages were difficult to find because of the speed with which cell walls were destroyed. In blocks attacked by *Stysanus*, there was an extremely sharp differentiation between rotted and unrotted zones of fibre-tracheids. Rotted zones were frequently associated with xylem vessels in which hyphae were abundant (Fig. 11). *Camarosporium* and *Cephalosporium* were far less active species. Slight sculpturing of the wall by lumenal hyphae of *Camarosporium* was observed when dense 'ropes' of hyphae were present. *Cephalosporium* hyphae formed fine cavities in the S_2 layer in fibre-tracheids on the exposed face. Cross-penetrating lateral hyphae were common. Such hyphae were indicative of incipient decay of Type 1 in birch or Scots pine infected by

Chaetomium. *Phoma* sp. listed as a decay species by Duncan (1960) was found to be inactive in pure culture in this investigation.

Discussion

The techniques used for these preliminary micromorphological studies on the mode of degradation of wood cell walls by soft rot fungi proved to be extremely satisfactory. Moreover, it is suggested that the use of small blocks (0·7 cm^3) from which sections can be cut directly and examined microscopically provides a simple, sensitive and rapid biological method for diagnosing the presence or absence of soft rot. This technique would be particularly suitable for screening a number of fungi to determine their potentiality for decay. Other techniques would be required for quantitative estimations of their activity.

The micromorphology of fungal decay of wood has been given relatively little attention. Some early observations were made on the action of Basidiomycetes on timber (Nutman 1929, Cartwright 1930, Proctor 1941). Proctor published a photomicrograph showing hyphae of brown rot fungi penetrating conifer tracheid walls at right angles to the long axis, thus initiating the characteristic bore-holes produced by these fungi. The lower Ascomycetes and Fungi Imperfecti have for many years been regarded as saprophytic contaminants on wood and the potentiality of these fungi as wood-rotting fungi only recently realised. A number of isolates belonging to the Ascomycetes (8) and Fungi Imperfecti (36) have been recorded as inducing soft rot (Savory 1954, Duncan 1960, Krapivina 1960, Da Costa and Kerruish 1963). While many of the identifications of these isolates remain tentative it is evident that the number of potential soft rot fungi is increasing with further investigation of these groups. As a result of the present study three identified species, *Coniothyrium fuckelii*, *Stysanus stemonitis* and *Camarosporium ambiens* and a species of *Sphaeronema* may now be added to the list of previous records.

A number of points of interest have emerged from the results of this investigation. The action of the soft rot fungi appears to be intermediate between that of the wood-rotting Basidiomycetes and the staining-fungi showing properties of both under certain conditions. The initial stages of hyphal penetration observed by Proctor (1941) for brown rot fungi are similar to those shown by *Chaetomium* in the first type of decay (Type 1). Similarities cease after the formation of the fine lateral cavity which brown rot fungi enlarged until the cavity is much wider than the hypha. In soft rot this cavity remained as a uniformly fine canal approximately 0·5 μ in diameter. In this respect the soft rot fungi resemble sap-stain fungi (Cartwright and Findlay 1958). Some workers (Krapivina 1960) claim that some staining fungi (*Ophiostoma* spp., *Trichosporium heteromorphum*) branch within the trachied walls forming a 'hammer shape' which suggests similarities with the T-shape branching observed in the present study.

The general dissolution of the wall of birch fibre-tracheids by lumenal hyphae of *Chaetomium* and *Coniothyrium* and the rapid destruction of the wall is reminiscent of the action of Basidiomycetes. One of the reasons for the susceptibility of birch is the failure of the fibre-tracheid wall to withstand enzymic attack by this means. It is evident that extracellular enzymic activity is shown by cells along the length of each hypha and need not be confined to the hyphal tip. Moreover, the work of Cowling (1963) suggests that enzymic action at a distance (in terms of hyphal diameter) from the hyphae is shown by Hymenomycetes attacking wood. In support of this it was found that in Scots pine tracheids or birch fibre-tracheids (Fig. 12) a hypha remaining in an axial position in a cavity within the wall continued to enlarge the cavity in a lateral direction for the complete width of the secondary wall and similarly in birch the lumenal hyphae does not remain in contact with the wall as degradation proceeds. On the other hand in the initial penetration of the wall by fine lateral branches and subsequent vertical penetration as in the first type of decay (Type 1), enzyme secretion appears to be concentrated at the hyphal tip. There is at present no evidence for plasmodesmata or any physical discontinuity of an appropriate magnitude in the secondary wall through which these hyphae could pass and penetration must therefore, be the result of enzyme action.

There is a marked difference between the effect of *Chaetomium* on transverse and tangential blocks of Scots pine (no attack after six weeks) and on similar blocks of birch (attack by means of lumen erosion evident after one week). The reason for this is not known, but is probably related to the different chemical nature of the three layers of the secondary walls of wood cells in the two species. Further work is necessary to substantiate the validity of this assertion.

When considering the progress of infection in the first kind of decay (Type 1), several observations invite further investigation. Bailey and Vestal (1937) showed that the soft rot cavities are aligned in the same helical arrangement as the cellulose microfibrils. The results of the present investigation have shown that the hyphae begin to degrade a path in the direction of the microfibrils in the S_2 layer immediately after the initial vertical branching. At this stage the arms were found to be placed at a slight but definite angle to the long axis of the tracheids, further vertical penetration always proceeding by apical enzymic activity of the penetrating hypha. These observations suggest that initially there is a preferential hydrolysis of the cellulose of one microfibril or bundle of microfibrils. This argument also presumes that extracellular fungal enzymes begin to degrade the cellulose molecules of microfibrils from one end, the majority of which are believed to be arranged parallel to the microfibril axis (Roelofsen 1959). Modern workers however are divided on this point, King (1963) and Nisizawa *et al* (1963).

The nature of the stimuli which cause a hypha (a) to penetrate the wall laterally and (b) to branch axially (vertically) in the wall are not known. In severely-rotted Scots pine fence posts hyphae were occasionally observed connecting a pit to a decay cavity. In laboratory experiments the hyphae of *Chaetomium* preferentially entered trachied walls in areas free of pits and apparently at random although they passed freely through the pit chambers from the lumen of one tracheid to the lumen of an adjacent tracheid. The deposits which form the 'warty' layer covering the S_3 layer of conifer tracheids (Liese, 1957) may be cytoplasmic remains and may provide an entry stimulus. These deposits were not visible at the magnifications used in this investigation. The nature of the stimulus which causes the tip of the lateral penetrating hypha to change its direction by 90° and branch simultaneously in the opposite direction seems most likely to prove to be a chemical or physical property of the microfibrils and perhaps also of associated substances (lignin and the hemicelluloses) in the wall. This type of fungal attack and the subsequent constricted cavity formation appears to be unique to lignified cellulose walls, and has not been recorded during microbial degradation of any of the many types of cellulose fibres. There is little evidence for any property of the wall which might account for this unusual mode of action. Hess *et al* (1957) observed a periodic change in electron density along the length of the microfibrils of synthetic cellulose fibres but not of wood fibres. On the other hand Wardrop (1964) has failed to detect this periodicity. Alternatively it is possible that the hypha may preferentially attack cellulose present in the amorphous state since this is chemically more easily hydrolysed than crystalline cellulose (Walseth, 1952). The cellulose of the microfibrils is made up of crystallites and amorphous cellulose. Ranby (1958) gives the length of the crystallites as *ca.* 600°A (0·06 μ) and the diameter as *ca* 100 A° (0·01 μ) while the bands of amorphous cellulose between the crystallites are even smaller. So, for example, at the point of contact of the tip of the lateral hypha which is barely reduced in diameter compared with the rest of the hypha (*ca* 0·5 μ in diameter) a large number of both crystalline and amorphous regions of cellulose will be exposed to the hyphal tip.

It is difficult to explain the preferential entry of hyphae of *Chaetomium* into the walls to give a decay of Type 1 when the radial face of both Scots pine and birch are exposed particularly as the same fungal species induced decay of Type 2 in transverse and tangential blocks of birch. Wardrop and Addo-Ashong (1963) have shown that radial walls contain more lignin than tangential walls. The lignin is presumed to be intimately associated with the cellulose of the microfibrils being present either as an incrusting substance or chemically bonded to the cellulose molecules. (Merewether, 1963.) When the radial face is exposed the more heavily lignified radial walls make up the greater part of the exposed wall surface and it might be expected that initially the wall resists lumen erosion and enzyme attack occurs only when a hyphal tip comes into physical contact with the cellulose of an exposed bundle of microfibrils. Once the wall was penetrated, it was found that several series of spiralling cavities were rapidly formed within the same cell wall and adjacent cell walls by lateral branches from hypha lying in an existing cavity or by hyphal processes from the apex of a cavity. The role of lignin in the degradation process is not clear. Although it is known that many soft rot fungi produce cellulases (Gascoigne and Gascoigne, 1960) little is known about the nature of other enzymes produced. Oxidising enzymes capable of attacking lignin must be produced by the fungi as shown by the fact that lateral hyphae pass through the primary wall—middle lamella complex and that the whole S_2 layer of birch may be destroyed, but the production of these enzymes is presumed to be low compared with the wood-rotting Basidiomycetes, particularly the white rot fungi which actively break down both cellulose and lignin.

Micromorphological observations such as those described require a parallel series of biochemical studies before a fuller understanding of the action of wood-destroying microfungi on cell walls is achieved.

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